# INFLAMMATORY BOWEL DISEASE

# Reversal of abnormal collagen production in Crohn's disease intestinal biopsies treated with regenerating agents

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**Background:** Crohn's disease (CD) is characterised by inflammation, muscle layer overgrowth, and collagenous fibrosis of the intestinal tract, with no effective therapy against collagen accumulation.
**Aims:** We quantified production of collagen in resection specimens from normal and CD patients and investigated the effect of regenerating agents (RGTAs) on collagen production. RGTAs are chemically substituted dextrans engineered to mimic the growth factor protecting effects of heparan sulphates. RGTAs have been shown to enhance tissue repair in various in vivo models and to modulate in vitro collagen phenotype differentially according to their structure.
**Patients:** We studied intestinal biopsies from two groups of CD patients: treated with glucocorticoids (CD-GC group: 10 patients) or not treated (CD group: seven patients), and from seven control patients.
**Methods:** After 24 hours of ex vivo incubation with ($^3$H) proline, collagen I, III, and V were extracted by pepsin and quantitatively separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Biosynthesis of each collagen type was quantified on radiolabelled isolated collagen.
**Results:** Total intestinal collagen production in CD patients compared with controls was increased up to 3.5-fold overall (p<0.001). In particular, collagen III biosynthesis was enhanced by 6.2-fold (p<0.001) in CD patients. In the CD-GC group, collagen production abnormalities were less marked. RGTAs added to the incubation medium in the CD group decreased total collagen production by 50% and decreased collagen III synthesis by 76%.
**Conclusion:** This finding offers a rationale for using RGTAs in the treatment of intestinal fibrosis in CD, thus opening up a potential new therapeutic field for this family of drugs.

C rohn's disease (CD) is a chronic progressive disease of the gastrointestinal tract characterised by transmural granulomatous inflammation and thickening of the bowel wall, with muscle layer overgrowth and collagenous fibrosis. Excess of fibrillar collagens occurs in all bowel wall layers.[1][2] Recent findings suggest that increased collagen production may be pivotal in the mechanism leading to intestinal fibrosis, stricture development, and intestinal obstruction.[3][4] Yet no medications effective against intestinal collagen accumulation are available, and surgery remains the main treatment for fibrosis related morbidity in CD.[1]

Quantitative and qualitative variations in collagen phenotype have been documented in CD patients with conflicting results.[5–8]

The situation is further complicated in the intestine by the heterogeneity of the cell population capable of producing collagens. Mesenchymal intestinal cells producing collagens include fibroblasts, smooth muscle cells, and myofibroblasts.[3][9] Our first objective was to investigate collagen production in specimens of entire intestinal tissue fragments rather than in isolated cells. We quantitated the ex vivo production of collagens by intestinal tissue biopsies collected surgically from CD patients treated or not with glucocorticoids. Control biopsies were macroscopically normal intestinal tissue fragments from patients with distal intestinal cancer.

Evidence that collagen, and collagen III in particular, overproduction is related to fibrosis[8] has prompted a search for components capable of selectively regulating the production of specific collagen types.[10] We have demonstrated previously that low molecular weight heparin specifically decreased collagen III production.[11] Recently, we developed a family of heparan sulphate mimetic polymers engineered to stimulate tissue repair and protection in vivo, which we called ReGeneraTing Agents or RGTAs. These polymers are obtained by chemical substitutions of dextran (see fig 1 for detailed structures). In several experimental models, they promote healing of various tissues, including muscle,[12] bone,[13] skin,[14] and intestine.[15] They also induce extracellular matrix remodelling by decreasing collagen production,[16][17] and collagen III production in particular, relative to other collagen types.[18] Because this effect on collagen synthesis may be associated with an antifibrotic activity, we studied the effects of RGTAs on collagen overproduction by intestinal tissue affected by CD.

## MATERIALS AND METHODS
### Source of tissue
Intestinal tissue was obtained from three groups of patients who required surgical intestinal resection (medical details on patients and precise tissue specimen origins are indicated in table 1). For CD patients, behaviour status was indicated according to the Vienna classification.[19] The CD group included patients with CD who were not receiving glucocorticoids (CD group); the CD-GC group comprised patients with CD who were receiving glucocorticoids at a mean dose of approximately 30 mg prednisone/day. Tissue specimens from

**RG1503**

C (62%)

O
RO
$^{-}O_3SO$

B (22%)

RO
HO

A (16%)

RO
$^{-}OOC\ CH_2O$

n/3

R

$\begin{cases} SO_3^- \ (65\%) \\ H \ (30\%) \\ CH2COO^- \ (5\%) \end{cases}$

**RG1192**

C (48%)

O
RO
$^{-}O_3SO$

D (23%)

RO
$XOOCH_2O$

B (13%)

RO
HO

A (16%)

RO
$^{-}OOC\ CH_2O$

n/4

X = NHCH₂ ⟨benzene⟩

R

$\begin{cases} SO_3^- \ (65\%) \\ H \ (30\%) \\ CH2COX \ (8\%) \\ CH2COO^- \ (6\%) \end{cases}$
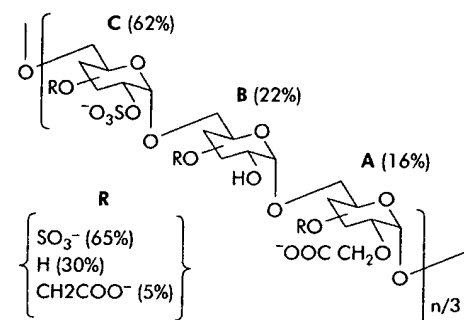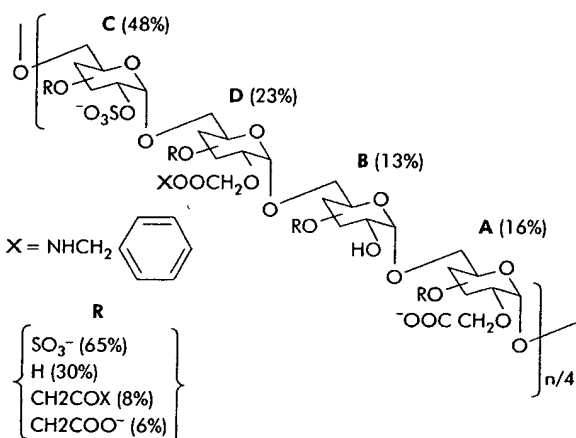
**Figure 1** Schematic structure of dextran derivatives. Polymers were elaborated from T40 dextran by chemical substitutions, as described in materials and methods. Dextrans were substituted by carboxy-methylation followed by O-sulphonation (RG1503) or by carboxy-methylation followed by amidation with benzylamine and O-sulphonation (RG1192). The different percentages indicated were calculated from the degree of substitution (ds) relative to the position of each group in a glucosidic unit, as reported in table 2. For an easy representation, the substituted glucosidic units A, B, C, and D were arranged in an arbitrary combination. Their respective proportions (%) within each polymer were calculated according to the nature of the group at the $C_2$ position. R is the proportion (%) of each substituted group at the $C_3$ plus $C_4$ positions.

patients in the CD and CD-GC groups were obtained from totally diseased areas with no portion of macroscopically normal tissue. In the control group, intestinal specimens were uninvolved areas of bowel removed from patients with distal intestinal cancer (at least 10 cm away from the tumour), with normal gross and histological features and no signs of inflammation.

### RGTA preparation

The water soluble dextran derivatives RG1503 and RG1192 (see fig 1 for detailed structures) were prepared from T40 dextran (average molecular weight ($M_r$) 37 000; Pharmacia, Paris, France) as previously described.[18 20 21] Briefly, for RG1503, carboxymethyl dextran was synthesised from dextran T40 by carboxymethylation of OH residues with monochloracetic acid treatment. The presence of carboxy-methyl groups was confirmed by infrared spectroscopy, which showed an absorption band at 1650/cm. Then, RG1503 was obtained from carboxymethyl dextran by O-sulphonation. The presence of sulphate groups was

indicated by infrared spectroscopy, with the appearance of two absorption bands at 1250 and 1025/cm, respectively.

For RG1192, the carboxymethyl dextran was dissolved in $H_2O/EtOH$, and carboxylate functions were activated with N-ethoxy-carbonyl-2-ethoxy-1,2- dihydroquinoline for 30 minutes at pH 3.5 and room temperature. This was followed by overnight incubation with 2 eq of free benzylamide. The product was precipitated and washed with methanol and dried under a vacuum. The infrared spectrum confirmed the presence of benzylamide with the appearance of a new absorption band at 1750/cm, corresponding to the carbonyl bond of the amide. RG1192 was synthesised from the derivatised carboxymethyl-benzylamide dextran by O-sulphonation. The presence of sulphate groups was indicated by infrared spectroscopy, with the appearance of two absorption bands at 1250 and 1025/cm, respectively.

Chemical characterisation of RG1503 and RG1192 was based on the degree of substitution (ds) of each individual group per glucosidic unit (table 2). A ds value of 3 indicates maximum substitution as one glucosidic unit contains three reactive OH groups at the $C_2$, $C_3$, and $C_4$ positions. Each ds value was determined by acidimetric titration and elementary analysis and confirmed by ^{1}H nuclear magnetic resonance. The distribution of the three reactive OH groups is reported in table 2. Average molecular weights of the RGTA molecules were estimated by high performance size exclusion chroma-tography, as described previously.[20] These polymers did not show significant anticoagulant activity (less than 5 IU/mg compared with 173 IU/mg for heparin).

### Tissue processing

Freshly resected tissue was placed in phosphate buffered saline and immediately transported to the laboratory for processing. In order to tentatively maintain uniformity in the sampling of tissue specimens, a standardised procedure was used to place each tissue specimen (approximately 1 g wet weight) on a cutting board and to slice it longitudinally into three pieces of similar macroscopic aspect and of approximately equal size.

### Intestinal collagen production

One of the three parts of each specimen was incubated in 3 ml of standard medium (Dulbecco's modified Eagle's minimal essential medium) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. The two other parts were incubated in 3 ml of standard medium with RG1503 (100 µg/ml) or RG1192 (100 µg/ml), respectively. Then, tissue specimens were labelled for 24 hours with (5-^{3}H) proline (25 µCi/ml, 30 Ci/ml; Amersham, France) plus ascorbic acid (50 µg/ml). At the end of the labelling period, the tissue was washed extensively with distilled water until no radioactivity was detected in the washing solutions.

### Collagen extraction, identification, and quantitation

Each tissue specimen (approximately 300 mg wet weight) was finely minced and homogenised. A small aliquot of homogenised tissue (10–15 mg) was hydrolysed in 6 M HCl at 105°C for 24 hours, for determination of total protein production, total collagen content, and total collagen production. Total protein production and total collagen production were determined by quantitation of (^{3}H) proline and hydroxy (^{3}H) proline by high performance liquid chromatography.[22 23] Total collagen content was determined by colorimetric hydroxyproline assay.

Then, all of the remaining minced tissue was digested with pepsin, as previously described.[22 24] Briefly, the tissue was dispersed in 0.5 M acetic acid containing pepsin (with a collagen to pepsin ratio of 10:1) for 24 hours at 4°C under

**Table 1** Intestinal tissue specimens included in the study

| Age (y) | Sex | Diagnosis | CD duration (y) | Surgical procedure | B/T |
|---|---|---|---|---|---|
| Control group | | | | | |
| 70 | F | Right colon cancer | | Right hemicolectomy | —/C |
| 62 | M | Right colon adenocarcinoma | | Right hemicolectomy | —/C |
| 55 | M | Small intestine carcinoma | | Ileum resection | —/I |
| 77 | F | Right colon cancer | | Right colectomy | —/C |
| 79 | M | Right colon adenocarcinoma | | Right hemicolectomy | —/C |
| 78 | F | Right colon cancer | | Right hemicolectomy | —/C |
| 51 | M | Colorectal cancer | | Proctocolectomy | —/C |
| CD group | | | | | |
| 48 | F | CD, fistulas | 3 | Ileocaecal resection | B3/O |
| 42 | F | CD, bowel obstruction | 3 | Ileocaecal resection | B2/O |
| 25 | F | CD, bowel obstruction | 6 | Ileocaecal resection | B2/O |
| 24 | F | CD, ileosigmoid fistula+bowel obstruction | 3 | Ileocaecal resection | B3/O |
| 21 | M | CD+bowel obstruction | 11 | Resection of middle portion of small intestine | B2/I |
| 26 | M | CD+bowel obstruction | 5 | Ileocaecal resection | |
| 21 | F | CD+bowel obstruction | 6 | Resection of small intestine | B2/I |
| CD-GC group | | | | | |
| 24 | F | CD, ileosigmoid fistula | 3 | Ileocaecal resection | B3/O |
| 29 | M | CD, right and transverse colon+bowel obstruction | 4 | Ileocolonic resection | B2/C |
| 27 | M | CD, bowel obstruction | 15 | Right ileocolonic resection | B2/C |
| 38 | M | CD, bowel obstruction | 9 mo | Ileocaecal resection | B2/O |
| 36 | F | CD, right and left colon | 6 | Ileocolonic resection | B1/C |
| 24 | F | CD, perineal lesions+bowel obstruction | 4 | Ileocaecal resection | B2/O |
| 28 | F | CD, bowel obstruction | 6 | Ileocaecal resection | B2/O |
| 34 | M | CD, bowel obstruction | 2 | Ileal and ileocaecal resection | B2/I |
| 20 | M | CD, bowel obstruction | 3 | Ileocaecal resection | B2/O |
| 30 | M | CD, bowel obstruction | 3 | Right colectomy | B2/C |

The control group is defined in materials and methods; CD group, patients with Crohn's disease not treated with glucocorticoids; CD-GC group, patients with Crohn's disease treated with glucocorticoids.
B, behaviour status (non-stricturing non-penetrating (B1); stricturing (B2), penetrating (B3)) according to the Vienna classification.
T, tissue origin of the portion of specimen studied (I, ileal; O, ileocaecal; C, colonic).

constant shaking. This procedure was repeated twice. The insoluble material was hydrolysed in 6 M HCl at 105°C for 24 hours, for determination of residual insoluble collagen (hydroxyproline). In all specimens, the amount of insoluble collagen was less than 10–15% of the total intestinal collagen.

Production of pepsin soluble collagen types was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).[22][24] Collagen bands were revealed by Coomassie blue staining and identified by comparison with standard collagen types (I, III, and V). Separation of collagen III was achieved by delayed reduction.[25] The relative proportions of radioactivity incorporated in collagens I, III, and V were quantified by excision of each individual collagen band followed by hydrolysis of the band in 6 M HCl at 105°C for 24 hours and by determination of hydroxy ($^3$H) proline in the hydrolysate.[22]

### Statistical analysis
For all parameters reported in the text and tables, results are expressed as the mean (SD) of independent determinations

carried out in triplicate on each tissue specimen with or without RGTA treatment. Differences between means in the two groups were evaluated using the Student's unpaired $t$ test. A p value <0.05 was considered statistically significant.

## RESULTS
### Collagen content
The collagen content of intestinal tissue from the CD, CD-GC, and control groups was determined by quantitation of hydroxyproline per unit of tissue wet weight. Total collagen content was significantly higher in the CD and CD-GC groups than in the control group (362 (44) (p<0.001) and 287 (32) (p<0.02), respectively, versus 183 (20) mg collagen/10 g tissue wet weight). Brief exposure (24 hours) of intestinal tissue to the RGTAs had no significant effect on total collagen content in any of the three groups.

### Collagen production
Each intestinal specimen was cut into three pieces: one was left untreated, one was treated with RG1503, and one was

**Table 2** Chemical characterisation of the regenerating agents (RGTA)

| ds* | | | | Position of groups expressed as ds | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | CM | | Su | | CMB | |
| CM† | Su‡ | CMB§ | H¶ | C₂ | C₃+C₄ | C₂ | C₃+C₄ | C₂ | C₃+C₄ |
| Polymer RG1192 (average Mr 140 000) | | | | | | | | | |
| 0.31 | 1.38 | 0.39 | 0.92 | 0.19 | 0.12 | 0.45 | 0.93 | 0.23 | 0.16 |
| Polymer RG1503 (average Mr 62 900) | | | | | | | | | |
| 0.26 | 1.92 | 0.82 | 0.16 | 0.10 | 0.62 | 1.30 | | | |

Chemical characterisation of each RGTA polymer is detailed in materials and methods.
*ds, degree of substitution of an individual group in one glucosidic unit (SD of ds values were less than 5%, with n=3).
†CM, CH₂COONa; ‡Su, SO₃Na; §CMB, CH₂CONHCH₂C₆H₅; ¶H, non-reacted hydroxyl groups; **C₃+C₄, global substitution at the C₃+C₄ positions calculated for each group as the difference between the total ds value and the ds value at the C₂ position.

Table 3  Effect of regenerating agents RG1503 and RG1192 on total collagen production in the bowel of patients with Crohn's disease (CD)

| | Protein production (total dpm/µg proline) | Collagen production (10² dpm hydroxy (³H) pro/mg collagen) |
|---|---|---|
| Control group | 12 900 (1900) | 1750 (220) |
| CD group | 15 800 (2600) | 6200 (890)*** |
| CD group+RG1503 | 13 800 (2200) | 3090 (470)†† |
| CD group+RG1192 | 15 900 (2400) | 5020 (780)** |
| CD-GC group | 14 200 (2100) | 3700 (560)‡ |
| CD-GC group+RG1503 | 14 300 (2300) | 2450 (380)‡ |
| CD-GC group+RG1192 | 13 700 (2200) | 3050 (420)* |

Control, CD, and CD-GC groups as described in table 1.
RGTA treatment of normal intestinal tissue (control group) did not significantly change total collagen production (data not shown).
*p<0.05, **p<0.01, ***p<0.001 compared with the control group.
†p<0.05 between RGTA treatment and no RGTA treatment of specimens from the CD group.
‡p<0.05 between RGTA treatment and no RGTA treatment of specimens from the CD-GC group.

treated with RG1192. After 24 hours of labelling with (³H) proline, total collagen production by the entire tissue fragment was determined (table 3). In the control group biopsies, RGTA treatment had no significant effect (data not shown). There were no significant differences in total protein production between the three groups. Total collagen production showed a marked increase in the CD group (by up to 3.5-fold) and a smaller increase in the CD-GC group (by up to 2.1-fold). Treatment with RG1503 significantly decreased total collagen production in the CD and CD-GC groups, with a return to control values in the CD-GC group. RG1192 had no significant effect on total collagen production in the CD and CD-GC groups. Collagen in each tissue specimen was solubilised by pepsin treatment which produced similar yields in all three groups (the amount of residual insoluble collagen was less than 10–15% of total intestinal collagen). Fibrillar collagen types were separated and quantified by SDS-PAGE. Table 4 shows that production of collagen types I, III, and V exhibited large increases in the CD group and smaller increases in the CD-GC group. Treatment of CD biopsies with RG1503 decreased collagen III production to

control values and also reduced production of collagens I and V. RG1192 decreased collagen III and collagen V production but had no effect on collagen I production. Treatment of CD-GC biopsies with RG1503 restored collagen I and III production to control values but induced a smaller decrease in collagen V production; RG1192 reduced collagen III and collagen V production to control values but did not modify collagen I production.

We compared the relative percentages of production of each collagen type in each group (table 4). Without RGTA treatment, the proportion of collagen III in the CD group was selectively increased compared with the control group. Interestingly, both RG1503 and RG1192 specifically decreased the percentage of collagen III production to control values. Note that the relative proportion of collagen V production was higher with RG1503. In the CD-GC group, the proportion of collagen III production without RGTA treatment was not significantly increased compared with control values. However, both RG1503 and RG1192 selectively decreased the proportion of collagen III production in this group.

Table 4  Effect of regenerating agents RG1503 and RG1192 on production of collagen types and on the relative production of collagen types in intestinal fragments from patients with Crohn's disease (CD)

| | Collagen production (10² dpm hydroxy (³H) proline/mg collagen) (collagen type production as % of total collagen production) | | |
|---|---|---|---|
| | Collagen I | Collagen III | Collagen V |
| Control group | 1300 (180) | 290 (40) | 80 (10) |
| % of total collagen | 77.8 (10.4) | 17.4 (2.4) | 4.8 (0.6) |
| CD group | 3840 (580)*** | 1800 (280)*** | 390 (60)** |
| % of total collagen | 63.6 (9.6) | 29.8 (4.6)* | 6.4 (0.9) |
| CD group+RG1503 | 2260 (360)*† | 430 (70)†††  | 260 (50)**† |
| % of total collagen | 76.6 (11.5) | 14.6 (1.9)†† | 8.8 (1.5)* |
| CD group+RG1192 | 3750 (450)*** | 690 (100)**†† | 230 (40)**† |
| % of total collagen | 79.9 (13.2) | 14.8 (2.2)†† | 5.2 (0.7) |
| CD-GC group | 2430 (370)* | 910 (140)** | 240 (40)** |
| % of total collagen | 67.9 (10.3) | 25.4 (3.8) | 6.8 (1.0) |
| CD-GC group+RG1503 | 1900 (280) | 230 (40)‡‡‡ | 160 (30)* |
| % of total collagen | 82.9 (12.5) | 10.9 (1.7)‡‡ | 6.9 (1.1) |
| CD-GC group+RG1192 | 2330 (350)* | 440 (80)‡‡ | 100 (20)‡‡ |
| % of total collagen | 81.1 (11.7) | 3.4 (0.5) | 15.5 (3.0)‡ |

Control, CD, and CD-GC groups as described in table 1.
RGTA treatment of normal intestinal tissue (control group) did not significantly change collagen type production (data not shown). The percentage of collagen type production compared with total collagen production was calculated from absolute values.
*p<0.05, **p<0.01, ***p<0.001 compared with the control group.
†p<0.05, ††p<0.01, †††p<0.001 between RGTA treatment and no RGTA treatment of specimens from the CD group.
‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001 between RGTA treatment and no RGTA treatment of specimens from the CD-GC group.

## DISCUSSION

CD is a severe intestinal disorder characterised by a chronic and unpredictable course with flares of acute inflammation separated by remissions.[2 6] The acute inflammation induces fibrosis with massive accumulation of a collagenous extracellular matrix. Onset of remission occurs when healing and repair mechanisms become operative. Thus antifibrotic drugs that enhance these mechanisms may hold therapeutic potential.

The goals of the present study were to improve the characterisation of collagenous fibrosis in human intestinal tissue from CD patients and to investigate the effect on this tissue of two antifibrotic compounds of the RGTA family, which promotes repair and remodelling of several tissues,[12–14] including the intestine.[15 27]

Our results indicate that alterations in collagen production associated with CD were not related to an overall alteration in protein production, which was not significantly modified (table 3).

Intestinal collagen production was increased 3.5-fold in biopsies from CD patients who were not taking glucocorticoid therapy. Our study indicates an important increase in collagen III production, which reached 6.2-fold (table 4) versus 2.9-fold and 4.8-fold for collagen I and collagen V, respectively. Increases in collagen III and also in collagen I production have been reported in fibrotic processes affecting various tissues,[8] including the intestine.[7 28]

CD patients who were taking glucocorticoid therapy (CD-GC group) had milder alterations in collagen production than patients in the CD group. The ability of glucocorticoids to decrease collagen production has been widely documented.[29–31] However, in addition to exerting desirable effects on collagen production and inflammation, glucocorticoids inhibit wound healing and tissue repair.

In the present study, very brief ex vivo exposure (24 hours) to RGTA partially or completely corrected collagen production abnormalities in intestinal tissue from CD patients. In particular, RG1503 reduced the increase in collagen III production in the CD group from 6.2- to 1.5-fold of control levels. It should be noted that RG1503 had an overzealous inhibitory effect on the percentage of collagen III in the CD-GC group, indicating that lower doses should be used in function of the present response of intestinal tissue.

In the CD-GC group, RGTA treatment almost completely restored the normal collagen expression phenotype group, suggesting that glucocorticoids and RGTA treatment may be complementary in the management of CD.

Differences were noted between the effects of RG1503 and RG1192. RG1503 was more effective in decreasing collagen I and III production and increased the percentage of collagen V in the CD group. RG1192 decreased the production of collagen III but not collagen I and had no effect on the percentage of collagen V. Differences in biological effects have been reported between RGTAs with or without benzylamide function substitution.[18 32] The activity of RG1503 is related mainly to interactions with transforming growth factor $\beta 1$ (TGF-$\beta 1$) whereas RG1192, which has a benzylamide function, could interact preferentially with fibroblast growth factor 2 (FGF-2).[18 32 33]

Given that the increase in TGF-$\beta 1$ content in the CD intestine is among the key factors involved in the development of fibrosis,[7] RG1503 might exert its activity on CD intestinal tissue by partly inhibiting or interfering with TGF-$\beta 1$ activity.

RG1192 mainly affected the proportion of collagen III production. We have found considerably greater affinity of RG1192 for FGF-2 compared with RG1503, as well as abolition by FGF-2 neutralising antibody of the effect of RG1192 on collagen III production (unpublished data). Thus

the effect of RG1192 on the CD collagen phenotype could be mediated by potentiation of the specific FGF-2 induced decrease in collagen III production.[34]

In addition, both RG1503 and RG1192 have been shown to inhibit the proliferation of various mesenchymal cells,[17 32 35] and thus RGTA may have beneficial effects on the abnormal cellular proliferation seen in CD.

Although the duration of tissue exposure to RGTA was extremely short in our study, the results suggest that these polymers may hold promise for correcting the collagen production abnormalities seen in CD, thus providing a noninvasive alternative to surgery. Regular heparin and low molecular weight heparin have shown some efficacy in treating the bowel inflammation seen in CD. However, treatment duration was kept short because of the risk of bleeding and the rationale for using heparin is unclear. RGTAs are heparan sulphates mimetic agents devoid of anticoagulant activity. Thus our findings indicate that further clinical research is warranted.

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